- **Claims**

1. A novel combination of 360 parameters for the production of transgenic tea (*Camellia sinensis* (L.) O. Kuntze) through biolistic which comprises

   (r) prior to the subjection of the leaf explants to 360 combinations, treatment of leaf explants with different concentrations (0.25-0.75M) of different osmotic agents ranging from sucrose, myoinositol, sorbitol, mannitol alone, combinations of mannitol and sorbitol and liquid basal MS medium (Murashige T. and Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497; 1962) supplemented with vitamins like thiamine-HCl (0.1 mg/l), pyridoxine-HCl (0.5 mg/l) and nicotinic acid (0.5 mg/l) together with glycine (2.0 mg/l) for different time periods ranging from 2 to 8 hrs

   (s) drawing of concentric circles of variable diameter ranging from 2.0-9.0 cm, on a transparent polythene sheet wherein the diameter of the outermost circle is same as that of a 9.0 cm petridish

   (t) arrangement of leaf explants with the adaxial surface up on the regeneration medium for bombardment

   (u) arrangement of leaf explants on the regeneration medium within the different concentric circles (2.0 to 5.0 cm) of 9.0 cm Petri-dishes for maximum spread of pRT99GUS plasmid DNA coated micro-projectiles produced by BioRad

   (v) sterilization of the gold particles by washing with 70% alcohol followed by sterile water for three times each

   (w) suspension of 60μg of gold particles in 0.5-1.5 ml sterile distilled water

   (x) dispension of 25-60μl of this suspension in 1.5 ml Eppendorf tubes for each bombardment

   (y) mixing of 50μl of gold suspension with 10μl of different concentrations of pRT99GUS plasmid DNA (0.5-5 μg/μl), 40-50μl of 1.5-3.5M $CaCl_2$ and 10-50μl of 0.5-2.0M spermidine free of phosphate salts with simultaneous vortexing from time to time, spinning for 5-20 seconds at 500-1100rpm followed by removal of the supernatant and washing with 70% ethanol and final suspension in 50-100μl of 100% ethanol

(z) coating of 10μl suspension of gold particles and DNA on sterile macro-carriers (BioRad) with immediate vortexing

(aa) development of 360 combinations comprising of: gap distances or distance between the rupture disc and the macro-carrier (1/4-3/8 inches alone and in combination), macrocarrier flight distance or the distance between macrocarrier and stopping screen (6-16 mm), and target distance or distance between the microprojectile stopping screen and target tissue (6-12 cm), for increasing the surface area for maximum particle penetration, minimum cell damage/injury and maximum regeneration efficiency

(bb) bombardment of leaf explants with biolistic guns like DuPont, Gene booster but specifically Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) under a chamber pressure of 22 to 28 inches mercury with gold particles (0.6-1.6μm), together with 1, 2 and 4μg/μl concentrations of DNA and the above 360 combinations wherein the tissue damage due to gas shock and high particle dispersion was circumvented by increasing the target distance for optimal particle dispersion and simultaneously the tissue damage due to off centred flight of microprojectile flight distance was overcome by decreasing the gap distance

(cc) bombardment of each plate twice after changing the direction of the Petri-plate by turning it by 180°

(dd) turning of the bombarded explants ranging from leaf, somatic embryos, zygotic embryos and embryogenic calli upside down on the regeneration medium with abaxial surface up such that the bombarded surface touches the regeneration medium

(ee) culturing in dark for two days at a temperature of 25+2°C of culture lab

(ff) assay of the bombarded leaves for GUS expression following the method of Jefferson, wherein the reaction of the GUS (5-bromo, 4-chloro, 3-indolyl, β-D-glucuronide) chemical with the transformed leaf explant produced a blue colour 'reaction product' thereby indicating the entry of the pRT99GUS plasmid DNA coated microprojectiles of BioRad into the cells of the explant tissue (Jefferson RA 1987, Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405)

(gg) transfer of the bombarded leaf explants after two days to the regeneration medium of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001under normal photoperiods of 16 h under cool fluorescent light of 52μmol $m^{-2}s^{-1}$ of the culture lab

(hh) finally selection of putative transformants after every 15 days on selection medium containing kanamycin (250-1100μg/ml) (r) regeneration of shoot buds from the completely folded, half opened or fully expanded leaf explants of 3 to 5 months old *in vitro* raised cultures following the protocol of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001 (s) growing and multiplying the transgenic shoots in liquid medium of Sandal I, Bhattacharya A, Ahuja P.S. An efficient liquid culture system for tea shoot proliferation Plant Cell Tissue Organ Culture 65(1): 75-80 (2001)(t) molecular characterization of GUS positive tissues of transgenic plants selected on 250-1100 μg/ml kanamycin using PCR and Southern Hybridization following standard methods.

2. A method as claimed in claim 1, wherein different explants like leaf, somatic embryos, zygotic embryos and embryogenic calli of different cultivars (*Chinary, Cambod and Assamica*) were genetically transformed through biolistics as stated above.
3. A method as claimed in claim 1, wherein leaf explants of *ex vitro* raised plants were treated with liquid basal hormone free MS medium and different osmotic agents wherein the least cumbersome and cheaper MS medium was most effective prior to bombardment with biolistic.
4. A method as claimed in claim 1 wherein leaf explants were treated with liquid basal hormone free MS medium and different ranges of osmotic agents like sucrose, myoinositol, sorbitol, mannitol alone and in combinations of mannitol and sorbitol wherein full strength hormone free basal MS medium was the most effective.
5. A method as claimed in claim 1 wherein the leaf explants were treated with hormone free liquid basal MS medium and different osmotic agents for different time periods

ranging from 2 to 8 hrs wherein hormone free liquid basal MS medium treatment for 4 hours was most effective.

6. A method as claimed in claim 1, wherein 50-70µg gold particle was prepared in sterile water both for direct use and storage in order to overcome the inhibitory effect of remnant glycerol during the loading of DNA onto the macro-carriers.
7. A method as claimed in claim 1 wherein concentric circles of variable diameter ranging from 2.0 to 9.0cm were drawn on a transparent polythene sheet where the diameter of the outermost circle was same as that of a 9.0 cm petridish.
8. A method as claimed in claim 1 wherein explants were arranged with adaxial surface up on the regeneration medium for bombardment.
9. A method as claimed in claim 1 wherein explants were arranged on the regeneration medium within the different concentric circles ranging from 2.0 to 5.0 cm of 9.0 cm Petri-dishes for optimization of the spreading pattern of pRT99GUS plasmid DNA coated micro-projectiles (BioRad) and using GUS assay method of Jefferson (Jefferson RA 1987, Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405).
10. A method as claimed in claim 1 wherein, gold particles ranging from 0.5-1.5 ml were sterilized by washing with 70% alcohol and sterile water for three times each.
11. A method as claimed in claim 1 wherein the suspension ranging from 25 to 60µl was dispensed in 1.5 ml Eppendorf tubes for each bombardment.
12. A method as claimed in claim 1 wherein, 40-60µl of gold suspension was mixed with 5-15µl of different concentrations of pRT99GUS plasmid DNA (0.5-5 µg/µl), 40-60µl of 1.5-3.5M $CaCl_2$ and 10-50µl of 0.5-2.0M spermidine free of phosphate salts.
13. A method as claimed in claim 1 wherein, the suspension was vortexed from time to time, with spinning for 5-20 seconds at 500-1100rpm followed by removal of the supernatant, washing with 70% ethanol and final suspension in 50-100µl of 100% ethanol.
14. A method as claimed in claim 1 wherein, 5-15µl suspension of gold particles and DNA were coated on sterile macrocarriers (BioRad) with immediate vortexing.

15. A method as claimed in claim 1 wherein, the explants were bombarded with biolistic guns like DuPont, Gene Booster and Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) but preferably Helium powered Particle Delivery system, PDS-1000/He (Bio-Rad) under a chamber pressure of 22 to 28 inches mercury.
16. A method as claimed in claim 1 wherein, 360 combinations were developed comprising of: gap distances or distance between the rupture disc and the macro-carrier (1/4-3/8 inches alone and in combination), macrocarrier flight distance or the distance between macrocarrier and stopping screen (6-16 mm), and target distance or distance between the microprojectile stopping screen and target tissue (6-12 cm), for increasing the surface area for maximum particle penetration, minimum cell damage/injury and maximum regeneration efficiency
17. A method as claimed in claim 1, wherein 360 combinations of the above together with gold particles ranging from 0.6 to 1.6μm, and concentration ranging from 1, 2 and 4μg/μl of DNA were used, wherein preferably a combination of 1.0 μm gold particles, 1100 psi burst pressure, target distance (9 cm), gap distance (3/8"+ ¼" and ¼"), macro-carrier flight distance (16 mm) and 1 μg/μl of DNA gave the maximum transformation frequency.
18. A method as claimed in claim 1 wherein, each explant was bombarded twice by changing the direction of the Petri-plates by 180° .
19. A method as claimed in claim 1 wherein, the bombarded explants were turned upside down on the regeneration medium with abaxial surface up.
20. A method as claimed in claim 1 wherein, the bombarded explants preferably leaf explants were cultured in dark for two days under culture lab conditions of 25+2°C followed by culture on regeneration medium of Sandal I, Bhattacharya A, Sharma M, Ahuja P.S. 'An efficient method for micropropagation of tea (*Camellia sinensis*) plants using leaf explants' patent filed in 2001
21. A method as claimed in claim 1, the bombarded explants were tested for transient expression using GUS assay method of Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol Biol Rep 5: 389-405 after 6 days of bombardment.

22. A method as claimed in claim 1, the leaf derived calli were selected after every 15 days on selection medium containing kanamycin.
23. A method as claimed in claim 1 wherein, kanamycin levels ranging from 250-1100$\mu g/ml$ kanamycin was used for selection of transformants almost no chance of 'escapes'.
24. A method as claimed in claim 1 wherein, 1.0 cm long healthy transgenic plants were grown and multiplied on kanamycin free liquid multiplication medium of Sandal I, Bhattacharya A, Ahuja P.S. An efficient liquid culture system for tea shoot proliferation Plant Cell Tissue Organ Culture 65(1): 75-80 (2001).
25. A method as claimed in claim 1 wherein, GUS positive tissues of transgenic plants selected on 250-1100$\mu g/ml$ kanamycin were characterized (molecular) using PCR and Southern Hybridization following standard methods.

ABSTRACT

The present invention relates to Production of transgenic tea (*Camellia sinensis* (L.) O. Kuntze) through biolistic